Appendix A

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MINIREVIEW
THE ROLE OF ANERGY IN PERIPHERAL T CELL UNRESPONSIVENESS

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Summary

When a T cell's encounter with specific antigen results in good signaling through the T cell antigen receptor yet does not lead to a proliferative response, the T cell enters a state of nonresponsiveness, or anergy. Anergy induction can result from a number of different situations, including antigen presentation by costimulation-deficient or "non-professional" antigen presenting cells, pharmacological blocking of T cell proliferation, or chronic stimulation of the T cell receptor by antigen. Anergy is a long-lived but temporary state characterized by a profound inability of the T cell to produce IL-2. Other effector functions may be affected to variable degrees. Anergy has been characterized most carefully under *in vitro* conditions, but several experimental models have demonstrated that T cells can also become anergic *in vivo*. This mechanism for tolerance induction may help to ensure that any mature autoreactive T cells which escape thymic deletion are unable to respond to host tissues. Furthermore, an understanding of the mechanism of anergy induction will most certainly lead to beneficial clinical applications, including improving graft acceptance and avoiding such deleterious immune responses as autoimmunity and allergy.

Key Words: anergy, T cell, IL-2

An effective immune system must be able to respond to infinitely diverse pathogenic microbes and foreign proteins and yet ignore the host's own potentially antigenic tissues. Because the consequences of an autoimmune response are potentially so severe, multiple mechanisms exist to ensure that autoreactive T cells are unable to mount an immune response. (Mechanisms also exist to control autoreactive B cells; for a review of B cell tolerance, see references 1 and 2). The major mechanism for establishing T cell self-tolerance is clonal deletion, or the physical elimination of self-reactive thymocytes early in their development via programmed cell death (apoptosis). This occurs when an immature T cell encounters specific peptide antigen bound to a major histocompatibility complex (MHC)-encoded molecule on the surface of a bone marrow-derived cell in the thymus. There is also evidence for nondeletional tolerance in the thymus, whereby immature T cells are made functionally nonresponsive, or anergic, to specific antigen. Because not all self antigens are expressed in, or gain access to, the thymus it is not surprising there are also tolerance mechanisms that act on mature T cells in the peripheral lymphoid tissues. Autologous cellular proteins are normally processed via the endogenous degradation pathway and presented by class I MHC molecules on the surfaces of all cells. Alternatively, extracellular autologous protein antigens that are shed or secreted from peripheral tissues can be endocytosed, degraded, and

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expressed on the cell surface in conjunction with class II MHC molecules. The priming and clonal expansion of class I MHC-restricted CD8+ T cells (but not their cytolytic function) requires that they receive CD4+ T cell-mediated help (i.e., IL-2) or a costimulatory signal mediated by CD80 on the APC interacting with CD28 on the T cell surface (3-5). CD80 expression is confined to bone marrow-derive"professional" antigen-presenting cells (APC) (i.e., dendritic cells, macrophages, and activated B cells). Because non-bone marrow-derived tissues are CD80-negative they would not be expected to prime a CD8+ T cell response. It is also unlikely that autoantigen-specific CD8+ T cells would receive help because maximal lymphokine production by CD4+ T cells requires both a costimulatory signal and antigen presentation by class II MHC molecules. Most peripheral tissues express neither costimulatory ligands nor class II MHC molecules, although class II expression can sometimes be induced under inflammatory conditions (6). Therefore, T cells specific for the self antigens expressed on parenchymal tissues are not normally activated, and these autoantigens are generally ignored. However, if shed tissue antigens or soluble protein antigens (i.e., peptide hormones, cytokines, or proteins expressed late in life) were taken up and processed by professional APC, it is certainly conceivable that an autoimmune reaction could result. Therefore, back-up mechanisms exist for directly silencing mature autoreactive T cells which may reach the peripheral tissues to ensure that autoimmune disorders remain relatively rare occurrences (reviewed in 7, 8). Tolerance mechanisms which appear to be operational in the periphery include (1) clonal deletion, (2) clonal anergy, and (3) immunosuppression, in which a T cell's response to antigen is inhibited by other T cells. This review will focus on T cell clonal anergy.

An historical perspective

T cell clonal anergy has been studied extensively *in vitro* using IL-2 producing T cell clones (known as Th1 cells) as a model system. We (9) found that peptide antigen presentation by splenic APC pretreated with carbodiimide (ECDI) not only failed to stimulate a proliferative response to peptide antigen, but also induced a state of unresponsiveness in the Th1 cells such that they were unable to proliferate in response to subsequent antigenic stimulation by normal APC. These anergic T cells remained viable, however, as shown by their ability to proliferate in response to exogenous IL-2. Furthermore, the unresponsive state appeared to be rather stable, as T cells restimulated as long as eight days later were still unable to respond to antigen. A similar state of unresponsiveness had been previously identified with MHC class II-restricted human T cell clones stimulated with soluble peptide antigen, presumably presented by class II-positive T cells (10). Numerous reports followed which documented that antigen presentation to purified T cell clones or freshly isolated T cells by resting B cells (11-13), purified class II MHC molecules in planar lipid membranes (14), keratinocytes (15, 16), granuloma (17) or thymic macrophages (18) also induced T cell anergy, as did stimulation of T cells (rigorously depleted of any APC) by anti-CD3 antibody (15) or the T cell mitogen concanavalin A (20). In each of these experimental situations, engagement of the T cell receptor (TCR)/CD3 complex resulted in T cell anergy rather than activation and clonal expansion. These results were consistent with the idea that TCR stimulation in the absence of an APC-derived "costimulatory" signal results in the induction of subsequent unresponsiveness. Support for this idea came from the finding that allogeneic APC that could not present peptide to the TCR on the T cell clone under study stimulated T cell proliferation and prevented the induction of T cell unresponsiveness by fixed APC and peptide (21). Dendritic cells were the most potent APC population in providing the costimulatory signal, while macrophages and activated B cells were less effective, and resting B cells were completely ineffective (22). The costimulatory ligands on the allogeneic accessory cells have now been identified as members of the B7 (CD80) family which bind to CD28 on the T cell (reviewed in 23-25). It is now clear that chemically fixed splenocytes are costimulation-deficient because the predominant APC in this population are resting B cells, which are CD80-negative, and fixation prevents the resting B cells from inducing CD80 expression.

Evidence that CD28 is an important costimulatory molecule in T cell activation came from studies demonstrating that anti-CD28 monoclonal antibodies, like allogeneic accessory cells, restore an IL-2-driven proliferative response to T cells stimulated by peptide antigen-pulsed, chemically fixed APC or anti-TCR antibody (reviewed in 23-25). T cells stimulated by TCR ligation alone do not produce detectable IL-2 and therefore cannot proliferate, unless cultured at very high density in which case some CD80+ T cells may themselves provide costimulatory signals (26). High levels

of IL-2, on the other hand, are produced with TCR ligation in the presence of CD28 costimulation. In contrast, anti-CD80 antibody blocks the delivery of costimulatory signals from viable APC (reviewed in 23-25), as does a soluble form of CTLA4, which is a CD28 analog and a second high affinity ligand for CD80 on activated T cells (reviewed in 25).

The addition of anti-CD28 antibody to stimulation cultures with peptide antigen-pulsed, chemically fixed APC not only restores an IL-2-driven proliferative response but also prevents anergy induction in murine T cell clones (27). Consistent with this is the observation that tetanus toxoid-specific human T cell clones proliferate in response to antigen presented by cells co-transfected with HLA-DR7 and B7, but antigen presentation by cells transfected with HLA-DR7 alone (i.e., in the absence of costimulation) results in T cell clonal anergy (28). It is evident, then, that ligation of CD28 by a monoclonal antibody mimics binding by the natural ligand CD80 present on allogeneic accessory cells, and interaction of the CD28/CD80 receptor/ligand pair is essential if anergy is to be avoided following TCR ligation.

The mechanism for CD28-mediated costimulation is not yet fully understood although recent observations have proved enlightening. CD28 signals through a biochemical pathway distinct from that used by the TCR/CD3 complex, as the hydrolysis of inositol phospholipids which occurs as a result of TCR engagement is not affected by costimulatory signals delivered through CD28 (M. Jenkins, unpublished observations). As further evidence for a novel signaling mechanism, CD28 synergizes with phorbol ester treatment to stimulate IL-2 production and proliferation by a cyclosporine A-resistant activation pathway; anti-CD3 antibody plus phorbol ester-induced activation, on the other hand, is sensitive to cyclosporine A treatment (reviewed in 25). Two groups have reported that CD28 may transduce its signal by activating a protein tyrosine kinase that phosphorylates a novel, unidentified 100 kDa protein (29, 30), although this kinase has not yet been identified. Phosphatidylinositol 3-kinase (PI 3-kinase), which is frequently a substrate for receptor-associated protein tyrosine kinases, becomes associated with CD28 following anti-CD28 stimulation of Jurkat T cells (31). Furthermore, B7-transfected Chinese hamster ovary (CHO) cells, but not parental CHO cells, induce elevated levels of the lipid products of PI 3-kinase activity, phosphatidylinositol (3,4)-bisphosphate and phosphatidylinositol (3,4,5)-trisphosphate, which is consistent with a potential role for this enzyme in CD28-mediated signaling (32). Finally, Su et al. (33) have recently reported that CD28 occupancy activates Jun kinase to phosphorylate and activate the transcription factor Jun, thereby enhancing its ability to augment transcription of the IL-2 gene.

The end result of CD28 signal transduction in the T cell that also receives TCR signals is both an increased rate of transcription of IL-2 mRNA (34) as well as an increase in its half-life (35). Increased IL-2 mRNA transcription most likely results from the activation of transcription factors, CD28RC and Jun, which bind to specific sequences within the IL-2 gene enhancer. These proteins appear only when the TCR and CD28 are both engaged. The binding of CD28RC to labeled CD28RE is inhibited by an unlabeled κB-like sequence (36) and thus may be related to the NF-κB transcription factor. Jun dimerizes with either itself or a member of the Fos family and then binds to the AP-1 target sequence in DNA. The increase in IL-2 mRNA stability seen with costimulation, on the other hand, most likely results from an interference with a degradation pathway. IL-2 mRNA has an inherently short half-life due to an AU-rich instability sequence in its 3'-untranslated region which targets it for rapid degradation. Other cytokine mRNAs which also contain this motif are similarly targeted for degradation and are also affected by costimulation.

Induction of the anergic state

The induction of anergy in murine Th1 clones has been extensively studied and shown to be dependent on a TCR-mediated increase in intracellular calcium. Stimulation by the calcium ionophore ionomycin alone is sufficient to induce anergy, and chelation of calcium ions by EGTA blocks anergy induction (37). In addition, anergy cannot be induced in the presence of cyclosporine A (19), an immunosuppressive drug which binds calcineurin to block calcium-dependent signaling events. The induction phase requires less than four hours of TCR stimulation in the absence of costimulation; if a costimulatory signal is delivered to CD28 after this time, the cells are no longer responsive (20; M. Jenkins, unpublished observations). Finally, anergy induction is blocked by cycloheximide, indicating a requirement for new protein synthesis (14).

The critical event which determines whether the outcome of a T cell's encounter with antigen is activation or anergy appears to be T cell proliferation. When Th1 cells are stimulated with antigen presented by effective CD80+ APC, they normally respond by producing high levels of IL-2 and undergoing multiple rounds of proliferation. After a period of rest, this population is subsequently able to mount a proliferative response when restimulated by antigen. Conversely, anergy is induced when Th1 cells receive TCR-mediated signals but do not proliferate. This occurs when T cells fail to receive requisite APC-derived costimulatory signals and are therefore unable to produce IL-2, as was the case in the *in vitro* studies described above with costimulation-deficient APC or stimulation by anti-CD3/TCR antibody. CD28-mediated costimulation antagonizes the induction of anergy only because it restores a good IL-2-driven proliferative response. Blocking costimulation with anti-CD80 antibody or CTLA4-Ig also induces anergy by inhibiting IL-2 synthesis. In another scenario, T cells stimulated by antigen and costimulatory APC become anergic when IL-2-driven proliferation is directly blocked by experimental manipulations. DeSilva et al. (38) demonstrated that proliferation of a Th1 clone in response to peptide antigen and costimulatory APC was inhibited in the presence of anti-IL-2 and anti-IL-2 receptor antibodies. Cells stimulated under these conditions become anergic even though they receive the necessary costimulus and produce IL-2. Similarly, Beverly et al. (39) reported that when a Th1 clone was fully activated with antigen and normal APC for 16-20 hours and then washed to remove all of the IL-2 they had produced, they too were unresponsive to further stimulation presumably because they required continuous exposure to IL-2 to recover responsiveness to antigen. Th1 cells stimulated with antigen and normal APC but directly blocked in their cell cycle progression at G_{1a} by the drug *n*-butyrate also become anergic (40). Sloan-Lancaster et al. (41) described a different kind of situation resulting in anergy. They demonstrated that an analogue of an immunogenic peptide could partially stimulate Th1 clones without inducing proliferation or cytokine production. The analogue induced anergy presumably by binding class II MHC molecules on functional APC and interacting with the TCR differently than the wild-type peptide (i.e., with a lower affinity). Finally, Th1 clones whose TCR are *chronically* stimulated with anti-CD3 antibody fail to proliferate in response to the IL-2 that they produce (42-44). Even in the presence of competent APC or anti-CD28 antibody, conditions which elicit high levels of IL-2 production, chronically stimulated T cells cannot proliferate and consequently become anergic (38). Therefore anergy appears to be induced in Th1 clones as a direct result of TCR stimulation in the absence of proliferation. These results are consistent with a model (39, 45) in which a TCR-mediated increase in intracellular calcium results in the production of a repressor protein. Under nonmitogenic conditions, the protein accumulates and reaches a concentration in the cell where it is then responsible for somehow maintaining the anergic state (e.g., by repressing the synthesis of IL-2). The amount of the anergy protein would presumably be reduced as a consequence of cellular division resulting in a concentration in the cell that is not biologically effective. There is indeed indirect molecular evidence for a negative regulatory protein which may repress transcription of IL-2 mRNA, as will be described later.

Murine Th2 and Th0 cells can also become anergic, as can CD8+ T cells, and the anergy-associated growth defect in these populations is different from that for Th1 cells. Several groups have been unable to induce proliferative anergy in Th2 clones (46, 47), and it has been hypothesized that Th2 cells may provide their own costimulatory function (48). For some IL-4 producing Th2 clones, however, APC-derived IL-1 can function as a costimulatory molecule by allowing the T cells to respond to the IL-4 they produce (49). McArthur and Raulet (50) demonstrated that CD28 costimulation of two Th2 clones induced increased proliferation through an increase in IL-4 responsiveness that was IL-1 dependent; i.e., CD28 costimulation enhanced IL-1 production. Gilbert et al. (51) have reported that Th2 cells stimulated in the absence of IL-1 enter an anergic state manifested by an inability to provide B cell help, although they do not lose their ability to proliferate in response to antigen. Anergy can also be induced in Th0 clones. Mueller et al. (52) have reported that anergy in dual IL-2/IL-4 producing Th0 clones is characterized not only by the IL-2 production defect seen with anergic Th1 cells, but also by an inability to respond to the IL-4 they produce.

The long-term T cell clones which have been used in many anergy experiments are by definition memory T cells, and there has been some question as to whether freshly isolated, naive T cells are capable of becoming anergic during their first encounter with antigen. In our hands, both murine

and human naive T cells have proven to be very difficult to anergize *in vitro*. Indeed, Davis and coworkers (53) found that although naive TCR transgenic T cells failed to respond to peptide antigen presented by purified MHC molecules, these cells did not become unresponsive to subsequent stimulation with peptide and APC. In contrast, Tan et al. (54) have demonstrated that freshly isolated human T cells become unresponsive when stimulated in a secondary mixed lymphocyte culture in the presence of CTLA4-Ig, a chimeric fusion protein that blocks costimulation by effectively competing with CD28 for the costimulatory ligand on allogeneic APC. Boussiotis et al. (55) reported that freshly isolated T cells become specifically unresponsive to antigen when preincubated with class II MHC+ cells that lack B7 but not when preincubated with class II MHC+ cells that express B7. Although it is possible that the unresponsiveness observed in these studies is anergy, it is impossible to conclude this because the fate of the alloantigen-specific T cells cannot be physically measured in a polyclonal T cell population. This problem can be circumvented by using stimuli that activate all T cells, such as anti-CD3 antibodies. Several groups have reported that freshly isolated T cells become unresponsive to subsequent restimulation if first cultured with anti-CD3 antibodies in the absence of B7+ APC (47, 56-58). Although many aspects are similar, the relationship of the unresponsive state achieved in these cells to that achieved in T cell clones is not clear. For example, when freshly isolated T cells are used, the induction of unresponsiveness is not always inhibited by costimulatory signals; in some cases it is actually enhanced, not blocked, by cyclosporin A (59); and unresponsiveness can occur following a proliferative response by the T cells (58). A potentially unifying explanation may be related to the length of time the T cells are exposed to the anergy-inducing stimulus. Many of the studies that employed resting T cells used long preincubation periods for inducing unresponsiveness. It is therefore possible that naive T cells may not be initially susceptible to anergy induction. Once activated, however, the T cells could become susceptible. Even if they proliferated initially, they would still be exposed to a TCR stimulus in the absence of costimulation which would then result in the induction of the unresponsive state later in the prolonged preincubation culture period.

The induction of peripheral anergy *in vivo*

Much of what we know about T cell anergy has come from *in vitro* models. The extent to which T cell self-tolerance for cellular antigens is maintained *in vivo* by clonal anergy in normal, unmanipulated individuals is not yet known. The difficulty in answering this question, of course, lies in the formidable task of tracking low frequency antigen-specific T cells.

However, there is abundant evidence for T cells existing in an anergic state in *in vivo* models using transgenic or radiation chimeric mice. In transgenic mice expressing the class II MHC I-E molecule exclusively on pancreatic β cells, Vβ5+ and Vβ17a+ T cells known to react with an I-E-associated superantigen are not deleted but are unresponsive to *in vitro* stimulation by anti-TCR antibody (60, 61). Dual expression of I-E and B7 transgenes in pancreatic β cells does not result in tolerance but instead results in insulitis and diabetes (62). Thus, as predicted by the *in vitro* models, antigen presentation by a B7-negative tissue results in tolerance whereas antigen presentation by a B7-positive tissue results in immunity. This is clearly not the only potential outcome, however, as transgenic expression of antigens in parenchymal tissues has resulted in autoimmunity, tolerance manifested *in vivo* but not *in vitro*, or antigen ignorance (reviewed in 63).

In a radiation bone marrow chimera model, clonal anergy has also been shown to mediate T cell tolerance to the mammary tumor virus-encoded superantigen Mls-1^a. Most T cells expressing the Vβ8.1 or Vβ6 chain in their TCR recognize the Mls-1^a superantigen, regardless of which α chain is expressed in the TCR. When Mls-1^b bone marrow cells are injected into irradiated Mls-1^a recipients, T cells bearing Mls-1^a-reactive TCR are not deleted but become anergic, probably during development in the thymus (64). Similarly, Rammensee et al. (65) demonstrated that injecting Mls-1^b mice with Mls-1^a spleen cells induces clonal anergy in peripheral Vβ6+ T cells. Later studies showed that the Mls 1^a-specific T cells initially expand *in vivo* and then many are deleted, leaving behind an unresponsive population (66, 67). This system has been used to show that IL-4-producing, superantigen-specific T cells are also susceptible to peripheral tolerance induction *in vivo* (68). Tolerance to the Mls-1^a superantigen in a line of mice transgenic for the TCR Vβ8.1 chain has been shown to be maintained by both clonal deletion and anergy; approximately 20-50% of peripheral CD4+ T cells in these mice are anergic in that they do not

proliferate *in vitro* in response to anti-TCR antibody (69). Fink and coworkers (70) have recently described a similar situation in normal mice. The failure of these T cells to respond to anti-TCR antibody rules out the possibility that they are not really tolerant but instead express a TCR α-chain that precludes superantigen recognition. In each of these experimental models, the specific superantigen inducing T cell anergy is expressed on the surface of a costimulation-deficient accessory cell (i.e., pancreatic β cells, thymic epithelial cells, or resting B cells) in association with class II MHC molecules.

It was possible that the ability of superantigens to induce functional T cell unresponsiveness was not generalizable to conventional protein antigens. However, it has been known for many years that certain routes of protein antigen administration, in particular routes that result in systemic antigen delivery under non-inflammatory conditions, not only fail to induce immunity but instead result in tolerance (71) To address the cellular mechanisms responsible for this phenomenon, we adoptively transferred a limited number of TCR-transgenic T cells to normal syngeneic recipient mice and tracked the fate of this small population *in vivo* with an anti-clonotypic TCR antibody following antigen injection (72). When antigen is injected in adjuvant subcutaneously, these adoptive transfer recipients mount a good primary response, and antigen-specific T cells proliferate in the draining lymph nodes, move into the lymph node follicles and then slowly disappear. Those few remaining are hypersensitive when restimulated *in vitro* with antigen, as would be expected of memory T cells. In contrast, when antigen is injected systemically -- either into the blood or intraperitoneally -- antigen-specific T cells proliferate for a short time, then rapidly disappear. Those few antigen-specific T cells which remain are hyporesponsive upon restimulation, characteristic of an anergic population. These results are consistent with a previous report by Kawabe and Ochi (67) that Vβ8+ T cells in mice injected systemically (intravenously) with *Staphylococcus aureus* enterotoxin B are clonally expanded before dying by apoptosis. Those which persist are unable to produce IL-2 or proliferate in culture in response to superantigen stimulation.

As previously discussed, it is likely that most autologous antigens expressed on peripheral tissues are simply ignored by the host's immune system, and the induction of anergy in autoreactive T cells serves only as a failsafe mechanism for avoiding autoimmunity. However, with soluble protein antigens such as polypeptide hormones or cytokines, or with tissue antigens shed into the bloodstream, T cell anergy may normally play an important role in maintaining self-tolerance. One cell type which may function to induce anergy to soluble protein antigens *in vivo* is the resting B cell. Resting B cells are CD80-negative, a poor source of costimulation (22), a poor stimulator in a primary mixed lymphocyte culture *in vitro* (73), and induce Mls-1a-specific clonal anergy in Vβ6+ T cells *in vivo* (65). Soluble protein antigens present in small amounts in peripheral circulation would be expected to be preferentially taken up and effectively concentrated for presentation by B cells bearing surface immunoglobulin receptors specific for the protein. Because the induction of CD80 on B cells requires activation via surface immunoglobulin cross-linking, and soluble protein antigens are unable to crosslink surface immunoglobulin receptors, resting B cells would therefore be expected to present antigenic peptides but not the necessary costimulatory signals to antigen-specific T cells. Bynon and Parker (74) tested this hypothesis and reported that intravenous injection of small amounts of protein antigen did indeed result in antigen-specific T cell unresponsiveness. Injection of larger amounts of the antigen, on the other hand, did not induce unresponsiveness, presumably because other CD80+ APC populations (i.e., dendritic cells or macrophages) were able to endocytose sufficient amounts of antigen for T cell priming. Thus, the APC population that initially gains access to the antigen may determine the type of response that is evoked.

Gilbert and Weigle (75) have recently reported that even when activated, B cells are capable of inducing antigen-specific T cell anergy. They found that B cells activated for 24 or 48 hours with anti-immunoglobulin treatment upregulated CD80 expression to high levels and became more potent stimulators of a primary mixed lymphocyte reaction. However, activated B cells were still ineffective at stimulating an antigen-specific proliferative response *in vitro*, and they induced anergy in Th1 clones. These investigators hypothesized that activated B cells still express inadequate levels of CD80, or perhaps lack another important molecule necessary for Th1 activation. Their results may explain the observation by Fuchs and Matzinger (76) that naive (but not memory) T cells presented with peptide antigen on either resting or activated B cells *in vivo*

become unresponsive to subsequent stimulation. It would appear that the ability of resting B cells (and perhaps activated B cells as well) to induce T cell unresponsiveness to soluble proteins would present a problem for the immune system in responding to pathogens. Foreign antigens, however, do not usually confront the immune system intravenously as soluble protein antigens. It is more likely that foreign antigens appear in a multi-valent display, as a part of infectious organisms rather than as soluble protein, and enter the body at sites (e.g., skin and lungs) where potent APC like dendritic cells and macrophages are situated to pick them up. In addition, many microbial substances (e.g., lipopolysaccharide) are excellent inducers of costimulatory activity in APC, ensuring that T cells activation will occur when these APC present antigen.

There is also evidence for the induction of anergy *in vivo* following chronic TCR stimulation, a condition where an IL-2 response defect prevents T cell proliferation regardless of APC potency and the presence of costimulatory ligands. For instance, T cells chronically exposed to high circulating levels of *Mycobacterium leprae* antigens in patients with lepromatous leprosy appear to be unresponsive to the antigen (77). This may also explain the anergic state induced *in vivo* following intravenous injection of anti-CD3 antibody (78), high doses of soluble antigen (79), or superantigens (66, 67).

Maintenance of the anergic state

The critical change characteristic of anergic Th1 cells is their inability to produce the autocrine growth factor IL-2, and they are unable to proliferate in response to antigen and normal APC because of this fundamental defect. Additional mechanisms exist to ensure that anergic T cells are unable to clonally expand, such as the inability of Th0 cells to respond to the IL-4 they produce (52). In addition, both anergic Th1 clones and anergic CD4+ T cells from mice tolerized *in vivo* to the minor lymphocyte-stimulating antigen Mls-1^a are unable to proliferate in response to APC-derived IL-12, although IL-12-enhanced production of IFNγ is still evident in anergic cells (80).

Although IL-2 production is severely diminished in anergic Th1 cells, other effector functions are variably affected. We have shown by Northern blot analysis that while anergic Th1 cells stimulated by anti-CD3 plus anti-CD28 antibodies do not produce detectable IL-2 mRNA, macrophage inflammatory protein-1α mRNA production is detected in anergic cells, although at only about 10% normal expression (M. Jenkins, unpublished observations). Anergic Th1 cells restimulated by antigen and APC also produce detectable though greatly reduced levels of IFN-γ and IL-3 (81). Go et al. (46) have reported that anergizing a Th1 clone with lytic ability results in its inability to produce IL-2 or proliferate in response to antigenic stimulation, yet the anergic cells are still able to lyse target cells bearing the same antigenic target to which they cannot mount a proliferative response. Similarly, Otten and Germain (82) reported that an IL-2 producing CD8+ clone stimulated by antigen-pulsed chemically fixed APC maintains its lytic capabilities yet loses its capacity to produce IL-2 in response to restimulation, a phenomenon they described as "split anergy". While this is consistent with the tight correlation between CD28-mediated costimulation and IL-2 synthesis seen in Th1 cells, it demonstrates that anergic T cells are not completely idle because other effector functions are regulated separately from IL-2-driven proliferation.

The molecular changes in anergic T cells responsible for the defect in IL-2 production are not yet clear, although several potential mechanisms have been eliminated. Although the TCR can be transiently down-modulated following receptor occupancy, TCR/CD3 expression returns to normal in anergic cells (14, 19, 69, 83). Anergic cells also express normal levels of CD4, LFA-1, ICAM-1 (81), and CD28 (D. DeSilva and M. Jenkins, unpublished observations) on their surfaces. In one report of cloned human T cells, however, Staphylococcus enterotoxin-induced anergy was associated with a down-modulation of the TCR and CD28 (84).

Mueller and colleagues (20, 81) reported that signal transduction in an anergic murine Th1 clone was unaffected at the level of inositol phosphate generation and concluded that the TCR on anergic cells appeared to be coupled to normal intracellular signaling pathways. Gajewski et al. (85), however, reported multiple TCR-associated signaling defects in anergic T cell clones, including elevated basal levels of intracellular calcium and phosphatidylinositol 1,4,5-trisposphate which failed to increase significantly with restimulation, as well as altered tyrosine phosphorylation patterns. One explanation for this discrepancy is that in the former studies, anergic cells were

examined following six days of rest in the absence of antigen, while anergic cells in the latter studies were used directly from the anergizing cultures (i.e., after only one to three days). T cells exhibit a transient desensitization following normal antigenic stimulation such that their TCR are refractory to further stimulation (86). It is therefore important to differentiate long-lasting anergy-associated signaling defects from changes associated with transient TCR desensitization. LaSalle et al. (87) have also found that anergic human T cells are defective in their release of intracellular calcium in response to either CD3 crosslinking or APC presentation of antigen. Similarly, Blackman et al. (88) demonstrated that autoreactive T cells present in the periphery of transgenic mice failed to exhibit an increase in intracellular calcium in response to TCR stimulation. In both studies, unresponsive T cells did proliferate normally in response to ionomycin plus phorbol ester, thereby bypassing the TCR-mediated signaling block and indicating a signaling defect proximal to protein kinase C activation and intracellular calcium release. Both anergic CD4+ and CD8+ T cells from transgenic mice have been reported to exhibit altered tyrosine phosphorylation patterns (89). Two protein substrates in particular, p38 and p75, are similarly affected in anergic Th1 clones (90). In addition, Gajewski et al. (85) observed an increase in tyrosine phosphorylation of phospholipase Cγ1 in anergic cells and questioned whether it was constitutively activated following anergy induction. Anergic Th1 cells also appear to have altered levels of two protein tyrosine kinases, specifically, reduced levels of $p56^{lck}$ and elevated levels of $p59^{fyn}$ (85, 91). These results suggest that defects in upstream signaling events which occur as a result of TCR engagement and which are normally required for inducing IL-2 gene transcription may be responsible for maintaining the anergic state.

Anergic T cells may also be defective at the level of transcription of the IL-2 gene. Specifically, the transcription factor AP-1, which binds one of the IL-2 enhancer elements, appears to be down-regulated. Kang et al. (83) have shown that anergic T cells fail to initiate transcription from a heterologous promoter under control of the 5' IL-2 gene enhancer or by a multimer of the AP-1 sequence within the enhancer. Furthermore, the protein complex that binds the AP-1 site, probably a c-fos/c-jun heterodimer, is induced more slowly when anergic T cells are restimulated than in normal T cells. These results are consistent with the model for anergy induction previously described and, in fact, suggest that one potential "anergy protein" may be an AP-1 binding repressor made during anergy induction which competes with AP-1 for binding within the IL-2 enhancer. This model would predict that if the "anergy protein" has a sufficiently short biological half life, or if its effective concentration is sufficiently diluted with successive rounds of cell division, then anergy could be reversed. This indeed appears to be the case.

Although anergic T cells fail to produce IL-2 when stimulated, Th1 clones which constitutively express the high affinity IL-2 receptor are still able to proliferate in response to exogenous IL-2 (7). Furthermore, anergic T cells stimulated with IL-2 and allowed to undergo several rounds of cell division are rescued from their anergic state (38, 39). AP-1 activity is also restored when anergic T cells are driven to proliferate *in vitro* (83). Anergy can decay spontaneously over time when Th1 clones are maintained in culture in the absence of further TCR engagement, although this is a slow process and appears to be only a partial reversal (39). These results demonstrate that the anergic state, while long-lasting, is not permanent and further support the model that anergy is maintained by a relatively stable negative regulatory factor whose effective biological activity is effectively diluted by cell division (39, 45).

In several experimental models, clonal anergy has also been shown to be reversed *in vivo*, once the source of specific antigen is removed. When radiated bone marrow chimeric mice are constructed by injecting Mls-1^b bone marrow cells into irradiated Mls-1^a recipients, most of the T cells bearing Vβ6+ TCR are not deleted but are found to be anergic. Furthermore, when these anergic T cells are then adoptively transferred into Mls-1^a recipient mice, where they are no longer exposed to superantigen, they regain their normal function (64). *In vivo* anergy reversal has also been demonstrated in mice made tolerant to the superantigen Staphylococcal enterotoxin B (92). Following injection, thymectomized mice exhibit a diminished response to the superantigen which is partially recovered after two months and at normal levels after four months (presumably after antigen clearance). There is also evidence that mature human T cells repopulating severe combined immunodeficient (SCID) mice enter a state of reversible anergy, perhaps following encounter with xenoantigens on peripheral tissues (93). The anergic T cells are refractory to stimulation by anti-

CD3 antibody but proliferate in response to exogenous IL-2. After four to six weeks of *in vitro* culture, they regain their proliferative response to anti-CD3 stimulation. In a transgenic mouse model, mature CD8+ T cells from female mice bearing a transgenic TCR specific for the male H-Y antigen can be made anergic by adoptively transferring them into male nude mice (94). The mechanism for inducing anergy in this system may be different, as it follows a brief period of activation and clonal expansion in the male recipients and is characterized by a decreased surface expression of the TCR and CD8 on the anergic CD8+ T cells. Nevertheless, the cells can also be rescued from anergy when transferred back into female recipients which lack the H-Y antigen (95). Finally, *in vivo* administration of IL-2 has been reported to rescue self-reactive T cells from an anergic state and to lead to autoimmune disease, presumably because autoreactive T cells are allowed to become functional (96). It should be mentioned that in each of the experimental systems described, it has been difficult to formally exclude the possibility that a minor population of nonanergic T cells is selectively expanded rather than a reversal of anergy in the anergic population.

Clinical applications

Regardless of whether anergy is normally an important mechanism for maintaining peripheral self-tolerance *in vivo* or an interesting phenomenon confined primarily to unusual experimental conditions, understanding the mechanism will undoubtedly lead to important anergy-based clinical therapies for controlling deleterious immune responses. Potentially important applications include the prevention of allograft refection and the prevention or cure of ongoing autoimmune diseases.

For instance, several investigators have achieved a partial donor-specific tolerance to allogeneic MHC molecules by intravenously injecting mice with splenocytes depleted of dendritic cells and monocytes (97, 98). The likely explanation is that class II MHC molecules on resting B cells, which predominate in this population, engage the alloreactive TCR but fail to provide costimulation and consequently induce anergy. It is essential in these experiments that adherent cells be depleted. The achievement of only partial tolerance, in fact, may be due to the presence of contaminating dendritic cells, which even in very small numbers are potent stimulators. These results probably explain the historic observation that donor-specific blood transfusions improve kidney allograft survival (reviewed in 99).

Transplantation tolerance can also be achieved by directly blocking the delivery of costimulatory signals *in vivo*. Proliferation of human T cells in either a primary or secondary mixed lymphocyte culture is blocked by the CTLA4-Ig chimeric fusion protein, and these T cells (naive or primed, respectively) exhibit an alloantigen-specific hyporesponsiveness when restimulated (54). Lenshow et al. (100) demonstrated that *in vivo* administration of CTLA4-Ig at the time of transplantation prevented rejection of xenogeneic pancreatic islet grafts. Treating the murine recipients with anti-human CD80 antibody also enhanced acceptance of human islet grafts, though this protocol was less efficient than CTLA4-Ig treatment. A likely explanation is that the anti-CD80 antibody (specific for human CD80) blocks activation only of T cells responding to direct presentation of human MHC-restricted antigen on human APC, while CTLA4-Ig (able to bind both murine and human CD80) also blocks the activation of T cells responding to human proteins shed and presented by mouse APC. Significantly, a second transplant from the same donor (but not a third party graft) was accepted without further CTLA4-Ig treatment. These results indicate that long-term tolerance was in fact achieved, and that graft acceptance was not due to graft adaptation (i.e., a down-modulation of donor MHC molecules and costimulation ligands due to the depletion of passenger leukocytes from the graft). Similarly, Turka et al. (101) administered CTLA4-Ig to mice and prolonged survival of cardiac allografts, but most grafts in this study were eventually rejected. This group has now combined donor-specific splenocyte transfusion (administered at the time of transplantation) and CTLA4-Ig treatment (given two days later) and achieved long-term acceptance of cardiac allografts (102). Furthermore, second cardiac allografts from matched donors were also well tolerated.

Adhesion molecules have also been targeted for intervention in achieving transplantation tolerance. CD2 and LFA-1, which function not only to increase T cell conjugate formation by interacting with their specific ligands on APC but also as signal transducing molecules, have been implicated in costimulation (reviewed in 103); the relative importance of each of these potential functions is a

matter of some debate. Boussiotis et al. (55) reported that transfectants co-expressing the human class II MHC DR7 molecule and ICAM-1 were able to stimulate T cell proliferation, although once the T cells were recovered and restimulated, they were found to be anergic. T cells stimulated by transfectants co-expressing DR7 and CD80, on the other hand, proliferated well and remained responsive to subsequent antigenic challenge. So while ICAM-1 may enhance a suboptimal T cell proliferative response to antigen, it appears that the T cells undergo insufficient proliferation to avoid becoming anergic. Nevertheless, antibodies to LFA-1 and ICAM-1, when given together, can induce long-term survival of cardiac allografts (104). In this model, mice which accepted cardiac allografts later accepted donor-syngeneic skin grafts but rejected third party skin grafts, indicating that allospecific tolerance had indeed been established. Similarly, treatment with antibodies to CD2 and the CD2 ligand (CD48) has been reported to lead to indefinite cardiac allograft survival (105). When long-term survivors of an allograft were given a second donor-specific graft, however, both the first and second grafts were rejected. These latter results are consistent with acute graft rejection occurring as a result of an alloreactive response to passenger leukocytes (especially class II+CD80+ dendritic cells) transplanted with the graft (usually class II-negative, CD80-negative). Treating graft recipients with antibodies which block either (1) the delivery of costimulatory signals or (2) adhesion and thus conjugate formation with antigen-specific T cells results in either T cell clonal anergy or T cell ignorance, respectively. In either case, if a T cell response is prevented until immunogenic passenger leukocytes are depleted, the graft will not be rejected. Transplantation of a second donor-specific graft carrying immunogenic passenger leukocytes would result in acceptance if T cells had been anergized (i.e., following CTLA4-Ig treatment) or rejection if T cells had simply been prevented from interacting with the first graft (i.e., following administration of antibodies to adhesion molecules).

Antigen-specific tolerization protocols have also been applied to the prevention of T cell-mediated autoimmune disease. Intraperitoneal injection of immunodominant peptides of myelin basic protein (MBP) into mice can prevent experimental autoimmune encephalomyelitis (EAE) and also block the progression of ongoing disease by inducing antigen-specific T cell unresponsiveness (106). Smilek et al. (107) were able to prevent EAE by subcutaneous immunization with a MBP peptide analog with a single amino acid substitution. The peptide analog bound class II MHC molecules better and stimulated encephalitogenic T cells *in vitro*, yet was nonimmunogenic and nonencephalitogenic *in vivo*. The mechanism for preventing autoimmune disease in this case is not known, although neither transferrable suppression nor competitive binding to class II MHC molecules appeared to be involved. Similarly, treating young nonobese diabetic (NOD) mice with the pancreatic β cell antigen glutamic acid decarboxylase can prevent spontaneous diabetes by tolerizing antigen-specific T cells (108, 109). Finally, Aichele et al. (110) demonstrated that viral-induced diabetes in a transgenic mouse model can be prevented by tolerizing the autoreactive T cells. In their studies, a single subcutaneous injection of a peptide corresponding to the immunodominant epitope of lymphocytic choriomeningitis virus glycoprotein (LCMV GP) in incomplete Freund's adjuvant primed mice such that when they were subsequently challenged with live virus, the virus was cleared. Multiple intraperitoneal injections of LCMV GP, however, resulted in antigen-specific tolerance. Mice challenged with live virus following intraperitoneal injection developed high titers, and their CTL showed no antigen reactivity *in vitro*. This tolerization protocol was then used to treat transgenic mice expressing LCMV GP exclusively in pancreatic β cells. These mice normally develop CD8+ T cell-mediated diabetes following viral infection, but intraperitoneal injection of the peptide prevented diabetes and autoimmune destruction of the β islet cells. Tolerance was antigen-specific, as a response to the LCMV nucleocapsid protein persisted. Whether tolerance in this system occurs as a result of clonal anergy or deletion is not known, although this group has previously reported that injection of the LCMV GP peptide into transgenic mice expressing TCR specific for the peptide results in both peripheral deletion and anergy of antigen-specific CD8+ T cells (111). A second approach to achieving nonresponsiveness has been through the oral administration of antigen. Oral tolerance is believed to result sometimes from CD8+ regulatory T cells (reviewed in 112), although there is also evidence that oral tolerance may result from T cell anergy (113). So by carefully selecting a tolerization protocol with the proper route of tolerogen administration, it appears likely that in the future, clinicians will be able to evoke an antigen-specific nonresponsiveness to treat T cell-mediated immune disorders such as autoimmune diseases and allergic responses.

References

1. C. C. GOODNOW. Annu. Rev. Immunol. 10 489-518 (1992).
2. G. J. V. NOSSAL. Adv. Immunol. 52 283-331 (1992).
3. M. AZUMA, M. CAYABYAB, D. BUCK, J. H. PHILLIPS and L. L. LANIER. J. Exp. Med. 175 353-360 (1992).
4. S. E. TOWNSEND and J. P. ALLISON. Science 259 368-370 (1993).
5. L. CHEN, S. ASHE, W. A. BRADY, I. HELLSTROM, K. E. HELLSTROM, J. A. LEDBETTER, P. McGOWAN and P. S. LINSLEY. Cell 71 1093-1102 (1992).
6. R. PUJOL-BORRELL, I. TODD, M. LONDEI, A. FOULIS, M. FELDMANN and G. F. BOTTAZZO. Mol. Biol. Med. 3 159-165 (1986).
7. V. GEENEN and G. KROEMER. Immunol. Today 14 573-575 (1993).
8. G. KROEMER and C. MARTINEZ-A. Res. Immunol. 143 335-340 (1992).
9. M. K. JENKINS and R. H. SCHWARTZ. J. Exp. Med. 165 302-319 (1987).
10. J. R. LAMB, B. J. SKIDMORE, N. GREEN, J. M. CHILLER and M. FELDMANN. J. Exp. Med. 157 1434-1447 (1983).
11. J. D. ASHWELL, M. K. JENKINS and R. H. SCHWARTZ. J. Immunol. 141 2536-2544 (1988).
12. K. M. GILBERT and W. O. WEIGLE. Cell. Immunol. 139 58-71 (1992).
13. R. M. STACK, D. J. LENSCHOW, G. S. GRAY, J. A. BLUESTONE and F. W. FITCH. J. Immunol. 152 5723-5733 (1994).
14. H. QUILL and R. SCHWARTZ. J. Immunol. 138 3704-3712 (1987).
15. A. A. GASPARI, M. K. JENKINS and S. I. KATZ. J. Immunol. 141 2216-2220 (1988).
16. V. BAL, A. McINDOE, G. DENTON, D. HUDSON, G. LOMBARDI, J. LAMB and R. LECHLER. Eur. J. Immunol. 20 1893-1897 (1990).
17. M. J. STADECKER, J. K. KAMISATO and S. M. CHIKUNGUWO. J. Immunol. 145 2697-2700 (1990).
18. S. JAYARAMAN, Y. LUO and M. E. DORF. J. Immunol. 148 2672-2681 (1992).
19. M. K. JENKINS, C. CHEN, G. JUNG, D. L. MUELLER and R. H. SCHWARTZ. J. Immunol. 144 16-22 (1990).
20. D. L. MUELLER, M. K. JENKINS and R. H. SCHWARTZ. J. Immunol. 142 2617-2628 (1989).
21. M. K. JENKINS, J. D. ASHWELL and R. H. SCHWARTZ. J. Immunol. 140 3324-3330 (1988).
22. M. J. JENKINS, D. R. DeSILVA, J. G. JOHNSON and S. D. NORTON. Adv. Exp. Med. Biol. 329 87-92 (1993).
23. M. K. JENKINS and J. G. JOHNSON. Curr. Opin. Immunol. 5 361-367 (1993).
24. R. H. SCHWARTZ. Cell 71 1065-1068 (1992).
25. P. A. LINSLEY and J. A. LEDBETTER. Annu. Rev. Immunol. 11 191-211 (1993).
26. M. AZUMA, H. YSSEL, J. H. PHILLIPS, H. SPITS and L. L. LANIER. J. Exp. Med. 177 845-850 (1993).
27. F. A. HARDING, J. G. McARTHUR, J. A. GROSS, D. H. RAULET and J. P ALLISON. Nature 356 607-609 (1992).
28. C. D. GIMMI, G. J. FREEMAN, J. G. GRIBBEN, G. GRAY and L. M. NADLER. Proc. Natl. Acad. Sci. USA 90 6586-6590 (1993).
29. P. VANDENBERGHE, G. J. FREEMAN, L. M. NADLER, M. C. [illegible]LETCHER, M. KAMOUN, L. A. TURKA, J. A. LEDBETTER, C. B. THOMPSON and C. H. JUNE. J. Exp. Med. 175 951-960 (1992).
30. W. LU, A. GRANELLI-PIPERNO, J. M. BJORNKAHL, C. A. PHILLIPS and J. M. TREVILLYAN. J. Immunol. 149 24-29 (1992).
31. K. E. TRUITT, C. M. HICHS and J. B. IMBODEN. J. Exp. Med. 179 1071-1076 (1994).
32. S. G. WARD, J. WESTWICK, N. D. HALL and D. M. SANSOM. Eur J. Immunol. 23 2572-2577 (1993).
33. B. SU, E. FACINTO, M. HIBI, T. KALLUNKI, M. KARIN and Y. BEN-NERIAH. Cell 77 727-736 (1994).
34. J. D. FRASER, B. A. IRVING, G. R. CRABTREE and A. WEISS. Science 251 313-316 (1991).
35. T. LINDSTEIN, C. H. JUNE, J. A. LEDBETTER, G. STELLA and C. B. THOMPSON. Science 244 339-343 (1989).